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IZVORNI ZNANSTVENI RAD
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Procjena biokompatibilnosti četiriju vrsta dentinskih adheziva kao sredstava za indirektno prekrivanje pulpe

Biocompatibility Evaluation of Four Dentin Adhesives Used as Indirect Pulp Capping Materials

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Sažetak
U mnogim slučajevima indirektno prekrivanje pulpe (IPP) prihvatljiva je terapija za trajne zube u slučaju njezine reverzibilne upale. Za IPP koriste se različiti lijekovi – od kalcijeva hidroksida i staklenog ionomera do dentinskih adheziva. **Svrha istraživanja:** Svrha ovog istraživanja *in vitro* bila je izmjeriti citotoksičnost u staničnoj kulturi, uspoređujući četiri adheziva: Xeno® V (XE), Excite® F DSC (EX), Adhese® OneF (AD) i Prime & Bond NT (PB). **Materijali i metode:** Adhezivi su primijenjeni u skladu s uputama proizvođača. Nakon 24-satne izloženosti procijenjena je vijabilnost stanica s pomoću fotometrijskog testa (MTT test). Podatci su podvrgnuti analizi varijance (ANOVA). **Rezultati:** Adhezivi čija je glavna komponenta bila 2-hidroksietil metakrilat (HEMA) pokazali su se manje citotoksičnima, a oni koji su u svojem sastavu imali monomer uretan-dimetakrilat (UDMA) bili su najcitotoksičniji. Učinci na vijabilnost statistički su između adheziva značajno varirali. **Zaključak:** Rezultati pokazuju da je Adhese® OneF najmanje citotoksičan od ispitanih adheziva i može se koristiti kao sredstvo za indirektno prekrivanje pulpe. No Prime & Bond NT u istim je uvjetima pokazao smanjenu biokompatibilnost.

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Uvod

Regeneracija pulpe moguća je u slučaju reverzibilnog pulpitisa, bilo da je uzrokovan karijesom, jatrogeno ili traumom (1). Svrha indirektnog prekrivanja pulpe (IPP) jest očuvanje vitalnosti zuba na kojemu se pojavio reverzibilni pulpitis ili duboki karijes bez izloženosti pulpe (2). Kako bi se osigurao uspjeh IPP-a, važno je ukloniti karijes s caklinsko-dentinskog spojišta i bočnih stijenki kaviteta da bi se osiguralo najbolje moguće brtvljenje između zuba i ispuna te tako spriječila mikropropusnost (3 – 5). U tom postupku kalcijev hidroksid i staklenoionomerni cementi koriste se kao podloge s dobrim rezultatima.

Adhezivi se također mogu koristiti za IPP. Jetkanje prije nanošenja adheziva olakšava otapanje dentina jer oslobađa čimbenike rasta koji stimuliraju osteoblastičnu aktivnost. To potiče odlaganje sklerotičnog/tercijarnog dentina, smanjujući njegovu propusnost (6). Prema Falsteru (2002.) (7), jetkanje kiselinom koncentracije od 10 posto ima sličan bakteriostatski i baktericidni učinak kao kalcijev hidroksid. Iako je ta koncentracija niža od one koja se obično koristi, Falster je utvrdio da to nije ugrozilo bakteriostatski i baktericidni učinak.

Korištenje samojetkajućih adhezivnih sustava ili adheziva s prethodnom jetkanjem kiselinom smanjuje rubnu mi-

Introduction

Pulp repair is possible whenever reversible pulpitis occurs, whether caused by caries, restoration, or trauma (1). The objective of indirect pulp treatment (IPT) is to preserve vitality of pulpally involved teeth challenged by either reversible pulpitis or deep caries without pulp exposure (2). To ensure the success of IPT, it is important to eliminate caries from the dentino-enamel junction and from the walls of the lateral cavity to obtain the best possible seal between tooth and restoration, thus preventing microfiltration (3-5). In this procedure, calcium hydroxide and glass ionomers are used as liner materials with good results.

Adhesive resin systems offer another IPT option. Etching before applying the adhesive will facilitate dentin dissolution, releasing growth factors that stimulate osteoblast activity. This causes the formation of sclerotic/tertiary dentin deposits, reducing dentinal permeability (6). According to Falster (2002), (7) acid etching at a 10% concentration, has similar bacteriostatic and bactericidal effects as calcium hydroxide. Although this concentration was lower than that usually used, Falster found that this did not compromise its bacteriostatic and bactericidal effects.

The use of either self-etching adhesive systems or adhesives with previous acid etching reduces marginal microfiltration and caries recurrence (8, 9).

kropropusnost i tako snižava mogućnost stvaranja rekurentnog karijesa (8,9).

No kad j riječ o IPP-u, povoljni klinički ishodi ne ovise samo o fizičkim i kemijskim svojstvima korištenog proizvoda, nego i o biokompatibilnosti adhezivnog sustava (10). Biološka kompatibilnost mora biti osnovno svojstvo bilo kojeg materijala koji se koristi u ustima, a to je osobito važno za adhezive koji se primjenjuju u neposrednoj blizini zubne pulpe.

Klinička istraživanja otkrila su razmjerno malo nepovoljnih bioloških učinaka pri primjeni adheziva izravno na dentin. No mnogobrojna istraživanja *in vitro* (11, 12) pokazala su da komponente adhezivnih smola mogu citotoksično djelovati na fibroblaste. Tkiva pulpe mogu se patološki promijeniti kad dođu u doticaj s adhezivima jer nepolimerizirani monomeri mogu prodrijeti kroz dentin i ući u pulpu (13). Smolasti kompozitni materijali u organskoj matrici sadržavaju citotoksične komponente kao što su monomeri i komonomeri (14). Restauracijski materijali obično sadržavaju hidroksietil-metakrilat (HEMA), trietilenglikol-dimetakrilat (TEGDMA), bisfenol-A-glicidil-metakrilat (Bis-GMA) i uretan-dimetakrilat (UDMA), a svi su pronađeni u vodenim ekstraktima dobivenima iz polimeriziranih materijala (15, 16). Pokazano je da dentinski adhezivi imaju različite razine citotoksičnosti nakon izlaganja od 24 do 72 sata kako slijedi (od najviše do najmanje toksičnog): Bis-GMA > UDMA > TEGDMA > HEMA (17). Čini se da HEMA i TEGDMA pokazuju *in vitro* manju citotoksičnost od Bis-GMA-e ili UDMA-e koje su hidrofobnije (18, 19). Adhezivni sustavi sadržavaju niz komponenti pa interakcije između njih mogu rezultirati različitim razinama citotoksičnosti koja može biti veća ili manja u odnosu na pojedinačne tvari (17, 20).

HEMA je čest sastojak dentinskih adheziva i nalazi se u koncentracijama koje variraju između 30 i 55 posto te ima ključnu ulogu u procesu impregnacije dentina (21). Zbog niske molekularne težine i relativne hidrofilnosti, HEMA se može širiti kroz rezidualni dentin i štetno djelovati na vitalnost odontoblasta te na fiziološku aktivnost pulpe (22).

Polimerizirane dentinske smole u vodeni medij oslobađaju TEGDMA-u u velikim količinama uzrokujući velik udjel njihovih neizreagiranih dvostrukih veza (23). TEGDMA čini od 25 do 50 posto sadržaja dentinskih adheziva (24). Zbog svojih lipofilnih svojstava može prodrijeti u citosol i membranske lipidne odjeljke stanica sisavaca, gdje ima mnogobrojne citotoksične učinke (25).

Druga zajednička komponenta dentinskih adheziva – kamforokinon, jest fotoinicijator koji se oslobađa nakon polimerizacije (26, 27). On nije dio polimernog lanca, pa tako udjel komponente koja nije uključena u polimerizaciju može izazvati oksidacijsko naprezanje, oštećenje DNK i citotoksičnost (27).

Na taj način citotoksičnost adheziva može varirati ovisno o omjerima tih komponenti i njihovu potencijalu da prodiru u dentin.

U ovom istraživanju *in vitro* korišteno je testiranje u indirektnom kontaktu za procjenu potencijalnih citotoksičnih učinaka na stanične kulture četiriju nedavno razvijenih adheziva u različitim razrjeđenjima.

In IPT, however, good clinical outcomes depend not only on the physical and chemical properties of the product used but also on the biocompatibility of the adhesive system (10). Biological compatibility must be a basic property of any dental material, and this is particularly relevant for adhesives used in cases involving proximity to dental pulp.

Clinical research has revealed relatively few adverse biological effects derived from applying adhesives directly to dentin. But numerous *in vitro* studies (11, 12) have found that the components of adhesive resins can have cytotoxic effects on fibroblasts. The pulp tissues may suffer pathological alteration when they come into contact with resin-composite adhesives, since uncured monomers can penetrate the dentinal tubules and thus reach the pulp (13). Resin composite materials contain cytotoxic components such as monomers and co-monomers in their organic matrix (14). Restoration materials commonly include two-hydroxyethyl methacrylate (HEMA), triethyleneglycol dimethacrylate (TEGDMA), bisphenol A-glycidyl-methacrylate (Bis-GMA), and urethane dimethacrylate (UDMA), all of which have been found in aqueous extracts taken from the cured restoration materials (15,16). It has been shown that dentin adhesives present differing levels of cytotoxicity after exposure times of 24 and 72 hours as follows (from most to least toxic): Bis-GMA>UDMA>TEGDMA>HEMA (17). HEMA and TEGDMA would appear to present less cytotoxicity *in vitro* than Bis-GMA or UDMA, which are more hydrophobic (18, 19). Adhesive systems contain a range of components, hence interactions between these may lead to varying levels of cytotoxicity that may be higher or lower than the individual substances alone (17,20).

HEMA is a frequent constituent of dentin adhesive agents, and is present at concentrations that vary between 30 and 55%, playing a key role in the process of dentin impregnation (21). Due to its low molecular weight and relative hydrophilicity, HEMA can spread through residual dentin, which may have harmful effects on odontoblast vitality, as well as physiological activity of the pulp (22).

Polymerized dental resins release TEGDMA into aqueous media in large quantities causing a high proportion of their unreacted double bonds (23). TEGDMA makes up 25-50% of the content of dentin adhesives (24). Because of its lipophilic characteristics, TEGDMA has a capacity of penetrating the cytosol and membrane lipid compartments of mammalian cells with a number of cytotoxic effects (25).

Another common component of dentinal adhesives – camphoroquinone (CQ) – is a photoinitiator that was released following the polymerization (26, 27). CQ is not a constituent of the polymer chain; hence a proportion of the component not involved in polymerization can provoke oxidative stress, DNA damage, and cytotoxicity (27).

In this way, the cytotoxicity of adhesives may vary depending on the proportions of these components and their potential to penetrate the dentin.

This *in vitro* study used indirect contact testing to evaluate the potential cytotoxic effects of four recently developed adhesives in different cell culture dilutions.

Materijali i metode

U istraživanju je korištena linija fibroblasta L929 (European Collection of Cell Cultures) u mediju za kultiviranje [Dulbecco's Modified Eagle's Medium (DMEM)] u kombinaciji s 10 % fetalnoga telećeg seruma (FCS) i antibioticima (penicilin 100 i.j./ml i streptomicin 100 µg/ml).

Metil-metakrilat (Merck, Darmstadt, Njemačka) korišten je kao pozitivna kontrola, a medij za kultiviranje bio je negativna kontrola.

Indirektna metoda (na temelju ekstrakata) korištena je prema ISO standardu 10993-5 (28). Pilot-istraživanjem potvrđena je prikladnost korištene metodologije i valjanost prototipova. Nakon odmrzavanja stanične linije provedeno je centrifugiranje 200 g tijekom 10 minuta, nakon čega su izbrojene stanice i usađene u posudu za kultiviranje od 75 cm^3 koja je zatim inkubirana u 7,5 % $C0_2$. Izmjeren je raspon osjetljivosti i prikazana je krivulja rasta. Na temelju dobivenih rezultata, odlučeno je da se kultivira 5000 stanica po stanici tijekom 24 sata.

Adhezivni postupak

Materijali su korišteni u skladu s uputama proizvođača:

- Materijal 1: Xeno® V
- Materijal 2: Excite® F DSC
- Materijal 3: Adhese® One F
- Materijal 4: Prime & Bond® NT

Korišteni materijali, njihov sastav i proizvođači navedeni su u tablici 1. Svaki materijal stavljen je na Petrijevu ploču, kratko osvijetljen i ostavljen da se stvrdnjava dva sata. Uzorci su pokriveni s 2 do 8 ml medija za kultiviranje bez fenolnog crvenila, pri omjeru volumena i površine po jedinici od 64 mm^2/200 ml i držani su 24 sata u CO_2 inkubatoru. Nakon toga određen je pH ekstrakta – svi su imali pH od 8,5. Zatim je ekstrakt svakog materijala aspiriran sterilnom štrcaljkom i filtriran kroz pore promjera 0,45 ml.

Materials and methods

The trial utilized the L929 fibroblast line (European Collection of Cell Cultures) in a culture medium (Dulbecco's Modified Eagle's Medium (DMEM)) combined with 10% of fetal calf serum (FCS) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml).

Methyl methacrylate (Merck, Darmstadt, Germany) was used as positive control, while the culture medium was used as negative control.

An indirect method (based on extracts) was used following ISO 10993-5 norms (28). A previous test was carried out to confirm the suitability of the methodology employed and the validity of the prototypes. After thawing the cell line, centrifugation was carried out at 200 g for 10 minutes, followed by cell counting and seeding in a 75 cm^3 culture flask, which in turn was incubated under 7.5% CO_2. The test sensitivity range was evaluated, and a growth curve plotted. Based on the results obtained, it was decided to culture 5000 cells per cell for 24 hours.

Adhesive procedures

Experimental procedures were carried out in triplicate with 6 wells per variable. Materials were used following the manufacturer's instructions:

- Material 1: Xeno® V
- Material 2: Excite® F DSC
- Material 3: Adhese® One F
- Material 4: Prime & Bond® NT

These materials, their compositions, and the manufacturer of each material are listed in Table 1. Each material was placed on a Petri plate, light-cured, and allowed to set for two hours. The samples were covered with 2-8 ml of culture medium, without phenol red, at a surface-to-unit volume ratio of 64 mm^2 / 200 ml, and were kept in the CO_2 incubator for 24 hours. After this period of time, the pH of the extracts was determined; all yielded a pH of 8.5. Afterwards, the extract of

Tablica 1. Materijali, proizvođači i sastav
Table 1 Materials, manufacturer and composition.

Dentinski adheziv • Dentin adhese	Proizvođač • Manufactured	Sastav • Components
Xeno V (XE)	DENTSPLY De Trey GmbH (Konstanz, Baden-Württemberg, Njemačka • Germany) samojetkajući • Self-adhesive	Bifunkcijski akrilati, kiseli akrilati, esteri fosforne kiseline, akrilna kiselina, voda, dl-kamforkinon, tercijarni butan, stabilizatori • Bifunctional acrylate, acidic acrylate, functionalized phosphoric acid ester, acrylic acid, water, dl-camphorquinone, tertiary butane, stabilizer.
Excite F DSC	Ivoclar Vivadent, Schaan, Lihtenštajn • Liechtenstein dvokomponentni adheziv • Two-step adhesive	HEMA, akrilat fosfonske kiseline, Bis-GMA, dimetakrilati, silicijev dioksid, etanol, katalizatori, stabilizatori • HEMA, phosphonic acid acrylate, Bis-GMA, dirue thacrylates, silica, ethanol, catalysts, stabilizers.
Adhese One F	Ivoclar Vivadent, Schaan, Lihtenštajn • Liechtenstein samojetkajući • Self-adhesive	Primer: akrilni eter fosfonske kiseline, bisakrilamid, voda, kamforkinon, stabilizatori. Bond: Bis-GMA, GDMA, HEMA, pirogeni silicijev dioksid, CQ, tercijarni amini, stabilizatori • Primer: acrylic ether phosphonic acid, bisacrylamide, water, Camphoroquinone, stabilizers. Bonding: Bis-GMA, GDMA, HEMA, fumedsilice, CQ, tertiary amine, stabilizers.
Prime & Bond NT/ NRC	DENTSPLY De Trey (Konstanz, Njemačka • Germany) dvokomponentni adheziv • Two-step adhesive	Adheziv: PENTA, UDMA, cetilamie hidrofluorid, aceton, nanopunila (amorfni silicijev dioksid 8 nm), stabilizatori • Adhesive: PENTA, UDMA, cetylamine hydrofluoride, acetone, nanofiller (amorphous silicon dioxide 8 nm), stabilizers.

S medijem za kultiviranje bez fenolnog crvenila i odgovarajućim ekstraktom, pripremljene su otopine 1/1 (100 % ekstrakta), 1/2, 1/4, 1/8 i 1/16 za svaki materijal i izmjerena je osmolarnost otopina. Te su otopine dodane stanicama 24 sata nakon kultiviranja. Također su dodane otopine metil-metakrilata od 10 %, 5 %, 2,5 % i 1,25 % koje su korištene kao pozitivne kontrole. Da bi se procijenio utjecaj pH na vijabilnost stanica, uključene su jažice s medijem za kultiviranje bez fenolnog crvenila koje su služile kao negativna kontrola zajedno s drugim jažicama s medijem za kultiviranje pripravljenim na pH 8. Ploče su zatim 24 sata inkubirane u 7,5 % CO_2. Provedeno je ispitivanje citotoksičnosti metil-tiazol-tetrazolija (MTT) (MTT, Sigma Chemical Co. St. Louis, MO, SAD), mjerenjem apsorbancije u čitaču na 570 nm, koristeći se valnom duljinom od 690 nm kao referencijom. Nakon 24 sata izmjeren je pH ekstrakta – kod svih je vrijednost pH iznosio 8.

Rezultate je tumačio tehničar koji nije znao koji su materijali bili uključeni u različite uzorke. Citotoksičnost je analizirana i kvantitativno (postotak preživljavanja u odnosu na kontrolu) i kvalitativno (morfologija stanice i sposobnost preživljavanja).

Statistička analiza

Podatci su podvrgnuti dvosmjernoj univarijantnoj analizi varijance (ANOVA), dopunjenoj ispitivanjem podudaranja parova uz korištenje metode najmanje statistički značajne razlike (LSD) s Bonferronijevom korekcijom.

Rezultati

Na slici 1. su postotci vijabilnosti pulpnih fibroblasta. Kvantitativni rezultati citotoksičnosti (postotak vijabilnosti u usporedbi s kontrolom) dobiveni za svaki materijal. Za sve materijale se vijabilnost stanica smanjivala kako se povećavala koncentracija ekstrakta. Nisu utvrđene statistički značajne razlike između koncentracija 1, 2 i 3. Nisu dobivene ni razlike između koncentracija 4 i 5, osim u slučaju adheziva Prime & Bond.

Od testiranih adheziva najmanje citotoksičan bio je Adhese, a slijede Excite, Xeno i Prime & Bond (najcitotoksičniji). Njihovi učinci na vijabilnost stanica varirali su uz statistički značajne razlike ($p < 0,001$).

Utvrđeno je da je pH 8 smanjio vijabilnost stanica i to za 40 posto u usporedbi s kontrolom (slika 2.).

U kvalitativnoj procjeni citotoksičnosti (u usporedbi s kontrolom), metil-metakrilat pokazao je učinak koji se očitovao zaokruživanjem stanica i nestankom stanične jezgre. U slučaju negativne kontrole (medij za kultiviranje), stanice su zadržale svoj karakterističan izduženi oblik, a jezgra je ostala intaktna. Općenito, testirani materijali sadržavali su zaobljene stanice kod kojih se dogodila degeneracija (slike 3. i 4.).

each material was aspirated with a sterile syringe and filtered through a pore diameter of 0.45 mm.

With culture medium without phenol red and the corresponding extract, 1/1 (100% extract), 1/2, 1/4, 1/8 and 1/16 dilutions were prepared for each material, and the osmolarity of the dilutions was measured. These dilutions in turn were added to the cells 24 hours after seeding of the latter in 96-well culture plates. Methyl methacrylate dilutions of 10%, 5%, 2.5% and 1.25% were also added and used as positive controls. To assess the influence of pH upon cell viability, wells containing culture medium without phenol red were included, which served as negative controls, together with other wells containing culture medium prepared at pH 8. The plates were then incubated under 7.5% CO_2 for 24 hours, and methyl thiazol tetrazolium (MTT) cytotoxicity assay was performed (MTT; Sigma Chemical Co. St. Louis, MO, USA), measuring absorbance in a plate reader at 570 nm, using a wavelength of 690 nm as reference. After 24 hours, the extracts' pH was measured: all presented a pH of 8.

The results were interpreted by a technician blinded as to which materials were involved in different samples. Cytotoxicity was analyzed both quantitatively (% viability with respect to control) and qualitatively (cell morphology and viability).

Statistical analysis

Data underwent two-way univariate analysis of variance (ANOVA), supplemented by equality of matched pairs testing, using the least significant difference (LSD) method, with Bonferroni correction.

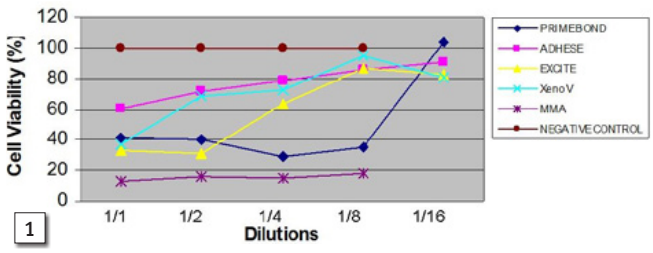
Results

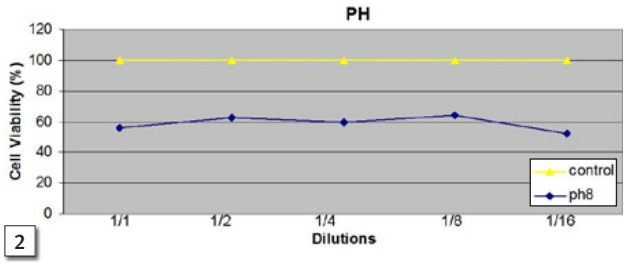
Figure 1 shows percentages of pulp fibroblast cell viability. Quantitative cytotoxicity results (% viability in comparison with the control) obtained for each material are expressed in Figure 1. For all materials, cell viability decreased as the concentration of extracts was increased. No significant differences were identified between concentrations 1, 2, and 3. Nor were differences obtained between 4 and 5, with the exception of Prime and Bond.

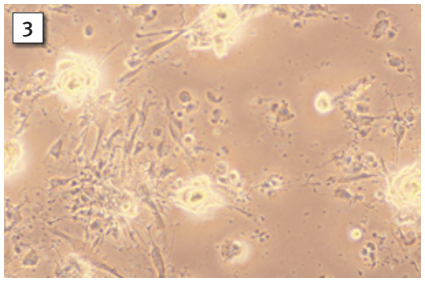
The least cytotoxic of the adhesives tested was Adhese, followed by Excite, Xeno and Prime and Bond (the most cytotoxic). Their effects on cell viability varied with statistically significant differences ($p<0.001$).

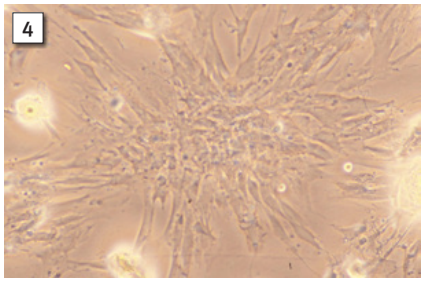
It was observed that pH 8 reduced cell viability, which was reduced by 40% in comparison with the control (Figure 2).

In the qualitative evaluation of cytotoxicity (compared with controls), methyl methacrylate had an effect evidenced by cell rounding and the disappearance of the cell nucleus. In the case of the negative control (culture medium), the cells were seen to maintain their characteristic elongated shape and the nucleus remained intact. In general, the materials tested included some rounded cells undergoing degeneration. (Figures 3 and 4).









Slika 1. Vrijednosti vijabilnosti stanica za sve materijale
Figure 1 Cell viability values for all materials.
Slika 2. pH vrijednosti
Figure 2 Cell viability pH values.
Slika 3. Stanična kultura nakon izlaganja. Materijal: Adhese® One F otopina 1/16.
Figure 3 Cell culture after exposition. Material: Adhese® One F 1/16 dilution.
Slika 4. Stanična kultura nakon izlaganja. Materijal: Prime & Bond® otopina NT 1/1.
Figure 4 Cell culture after exposition. Material: Prime & Bond® NT 1/1 dilution.

Rasprava

Biocompatibilnost je osnovno svojstvo svakoga dentalnog materijala, a posebno je značajna u slučaju dentinskih adheziva koji se nalaze u neposrednoj blizini pulpe. Smolasti monomeri i druge komponente adhezivnih sustava mogu uzrokovati različite razine oštećenja stanica zbog razlika u kemijskom sastavu (29). Interakcije između tih komponenti i dentina potiču različite reakcije pulpnog tkiva (30). Tako testirani adhezivi imaju različite razine citotoksičnosti, vjerojatno zbog varijacija u kemijskom sastavu, fizičkim svojstvima i načinu primjene.

Pri procjeni citotoksičnosti u istraživanjima se koriste različite metode kontakta stanica i materijala (31). Za ispitivanje citotoksičnosti Međunarodna organizacija za normiranje (ISO) (28) preporučuje uporabu etabliranih staničnih linija, uključujući L-929, Balb/3T3 i WI-38. One imaju homogenu morfologiju i svojstvo rasta te tako olakšavaju ponovljivost u ispitivanju citotoksičnosti *in vitro* (32). U ovom istraživanju odabrana je stanična linija L-929 jer je dostupna, često se upotrebljava u sličnim istraživanjima i ima povoljna svojstva u situacijama *in vitro*.

Materijali za indirektno prekrivanje pulpe nisu u izravnom doticaju s pulpom pa se primjenjuje ispitivanje u indirektnom kontaktu jer omogućuje realnije *in vitro* uvjete analize citotoksičnosti adheziva. Toksični učinci na stanice procijenjeni su MTT testom. Ta analiza reducira metil-tiazol-tetrazolij u obojeni formazan. Boja reagira na čimbenike koji inhibiraju aktivnost dehidrogenaze (33, 34).

Chen i suradnici (35) utvrdili su da su u pulpnim stanicama u kontaktu s adhezivima nastali različiti citotoksični učinci tijekom 24 sata, te da je to ovisilo o razrijeđenosti adheziva, tako da se citotoksičnost povećavala proporcional-

Discussion

Biocompatibility must be a fundamental property of any dental material and this is of particular relevance in the case of dentin adhesives in close proximity to the pulp. Resinous monomers and other components of adhesive systems can cause varying levels of cell damage due to differences in chemical composition (29). Interactions between these components and dentin will lead to varying pulp tissue responses (30). In this way, the evaluated adhesives produced different levels of cytotoxicity, probably due to variations in chemical composition, physical properties, and the method of application.

When evaluating cytotoxicity, research has employed different methods of cell-to-material contact (31). For cytotoxicity testing, The International Organization for Standardization (ISO), (28) recommends the use of established cell lines including L-929, Balb/3T3 and WI-38. These offer homogeneous morphology and growth characteristics and so facilitate reproducibility in *in vitro* cytotoxicity testing (32). The present study selected the L-929 cell line as it is readily available, has been widely used in similar research, and behaves efficiently in *in vitro* situations.

Indirect pulp capping materials do not enter into direct contact with the pulp; therefore, indirect contact testing was used, since it could provide more realistic *in vitro* conditions for testing the cytotoxicity of the adhesives. The toxic effects on cells were evaluated using the MTT assay. This assay reduces methyl thiazol tetrazolium metabolically to colored formazan; the color reacts to the factors inhibiting dehydrogenase activity (33, 34).

Chen *et al.* (35) observed that adhesives might cause cytotoxicity in pulp cells when they came into close contact for 24 hours, which depended on their dilution, hence cytotox-

no njihovoj koncentraciji. Citotoksični učinci smanjivali su se razrjeđenjem materijala zbog sve manje koncentracije toksičnih sastojaka.

Dentinski adhezivi sadržavaju različite kombinacije i različite koncentracije metakrilatnih monomera: Bis-GMA-e, HEMA-e, UDMA-e i PENTA-e. Varijacije u njihovoj koncentraciji utječu na toksičnost svakog materijala. Analize citotoksičnosti akrilata i metakrilata u dentalnim materijalima pokazuju različite vrijednosti koje ovise o strukturi (36). TEGDMA, Bis-GMA i UDMA imaju umjerenu razinu citotoksičnosti (36, 27). Ratanasathien i suradnici (17) ispitivali su citotoksičnost sastojaka dentinskih adheziva i rangirali su toksičnost od najviše do najniže kako slijedi: Bis-GMA > UDMA > TEGDMA > HEMA nakon 24 sata i 72 sata izloženosti. Kusdemir i suradnici(10) također su izvijestili da je primer korišten s dvokomponentnim samojetkajućim adhezivom temeljenim na HEMA-i imao nižu razinu citotoksičnosti od jednokomponentnog bonda koji sadržava monomere veće molekularne težine.

Dosadašnja istraživanja pokazala su da se tipične komponente adheziva i materijala za ispune, kao što su HEMA i TEGDMA, mogu širiti kroz dentinske tubule i prodrijeti u pulpu u koncentracijama milimola (13, 18). *In vitro* se pokazalo da čak i pri netoksičnoj razini ti monomeri mogu poremetiti normalne postupke diferencijacije pulpnih fibroblasta (13, 18). To otkriće u skladu je s rezultatima još jednog istraživanja koje je potvrdilo da su HEMA i TEGDMA štetne za diferencijaciju matičnih/progenitorskih stanica u odontogene, što negativno utječe na homeostazu i regeneraciju pulpnog tkiva (37 – 40). U dubokim kavitetima rezidualni monomeri mogu stići do pulpe difuzijom i lakše prodrijeti kada je dentin najetkan. U određenim koncentracijama oni toksično djeluju na stanice pulpe, što rezultira upalom i disorganizacijom tkiva. Pulpne reakcije variraju ovisno o dodatnim čimbenicima, uključujući sastav materijala i primijenjene kliničke tehnike (41).

U ovom istraživanju Adhese je imao nisku toksičnost, što je u skladu s rezultatima drugih istraživanja (42). To je zbog HEMA-e u sastavu. Bis-GMA ima najveću toksičnost među Adheseovim komponentama, ali i manji kapacitet prodiranja u dentin zbog veće molekularne težine (28, 29). No Bis-GMA podliježe hidrolizi, stvarajući metakrilnu kiselinu kao metabolit topljiv u vodi (MAA). MAA je izvor citotoksičnosti jer može stimulirati otpuštanje TNF-a, ili mijenjati lipidni sloj staničnih membrana, a to utječe na propusnost membrane (43).

UDMA je toksičnija za stanice od HEMA-e. Huang i Chang (29) utvrdili su veću citotoksičnost Prime & Bonda, što objašnjavaju prisutnošću UDMA-e u sastavu. I u ovom istraživanju adhezivi koji sadržavaju UDMA-u pokazali su se više citotoksičnima, pri čemu je Prime & Bond najcitotoksičniji. Ovaj rezultat slaže se s drugim istraživanjima koja su pokazala da je Prime & Bond početno vrlo citotoksičan (10).

U ovom radu nije pronađena značajna razlika između adheziva Xeno i Excite. Takav nalaz ne podudara se s drugim autorima (11) koji su uočili da Xeno pokazuje manju citotoksičnost jer ima manju tendenciju degradacije, više stabilnih molekula i ne sadržava HEMA-u ili Bis-GMA-u.

icity increased in proportion to concentration of the adhesive. In this way, cytotoxic effects decreased when materials were more diluted due to the reduced concentration of toxic constituents.

Dentin adhesives contain different combinations and different concentrations of four methacrylate monomers: Bis-GMA, HEMA, UDMA and PENTA. Therefore, variations in concentration affect the toxicity of each material. Evaluations of the cytotoxicity of acrylates and methacrylates in dental materials display varying levels of cytotoxicity that is dependent on structure (36). TEGDMA, Bis-GMA, and UDMA all present moderate levels of cytotoxicity (36, 27). Ratanasathien *et al.* (17) evaluated the cytotoxicity of the constituents of dentin adhesives and rated toxicity levels from highest to lowest as follows: Bis-GMA>UDMA>TEGDMA>HEMA after 24 hours and 72-hours exposure. Kusdemir *et al.* (10) also reported that the primer used with HEMA-based two-step self-etching adhesives presented lower levels of cytotoxicity than one-step bond materials containing monomers of higher molecular weight.

Previous studies have shown that typical components of adhesive and restoration resins, such as HEMA and TEGDMA are able to spread through dentin tubules, thus reaching the pulp tissue at concentrations that fall within the millimolar range (13, 18). It has been demonstrated *in vitro* that even at non-toxic levels; these monomers can disrupt the normal differentiation processes of pulp fibroblasts (13, 18). This finding concurs with another study that affirmed that HEMA and TEGDMA are detrimental to odontogenic differentiation of pulp stem/progenitor cells, an effect that would negatively affect the pulp tissue homeostasis and repair (37-40). Applied to deep cavities, these residual monomers can reach the pulp by diffusion, and penetrate more easily when the dentin has been etched. At certain concentrations they exert a toxic effect on pulp cells, resulting in inflammation and tissue disorganization. Pulp reactions vary in severity depending on additional factors including the composition of the material and clinical techniques employed (41).

In the present study, Adhese showed low toxicity, a finding that concurs with other research (42). This is due to the presence of HEMA in its composition. Bis-GMA shows the highest toxicity among components of Adhese but has less capacity to penetrate the dentin due to its higher molecular weight (228.29). However, bis-GMA is subject to hydrolysis, generating the water-soluble metabolite methacrylic acid (MAA). MAA is a source of cytotoxicity as it can stimulate TNF-α release, or alter the lipid layer of cell membranes, thus influencing the permeability of the membrane (43).

UDMA is more toxic to cells than HEMA. Huang and Chang (29) observed a higher cytotoxicity with Prime and Bond and argued that this is caused by the presence of UDMA in its composition. Indeed, in the present study, adhesives containing UDMA were found to be more cytotoxic, with Prime and Bond the most cytotoxic of all. This result agrees with other studies that have shown that Prime and Bond is initially highly cytotoxic (10).

The present study found no significant difference between Xeno and Excite. This finding is not in agreement with

Kamferokinon, kao najčešće korišteni fotoinicijator, može biti još jedan uzrok citotoksičnosti dentinskih adheziva (44). Ta tvar nalazi se u svim ispitanim materijalima (tablica 1.) i mogla bi utjecati na metabolizam stanica, mogući mehanizam koji izaziva negativne kliničke i subkliničke odgovore (42).

Uzimajući u obzir kontrolne uzorke, pH je bio nespecifična varijabla koja je utjecala na ukupnu vijabilnost stanica, tako da je ona (djelomično) smanjena zbog ovog čimbenika, a ne samo zbog specifične toksičnosti materijala. Zato bi dobiveni rezultati mogli imati veću pouzdanost kontroliranjem ove varijable.

Debljina dentina utječe na koncentraciju i količinu adheziva koji prodire u pulpu. Hamid i Hume (45) istražili su utjecaj debljine dentina na stupanj penetracije monomera u adhezivima nakon 24-satne inkubacije, testirajući dentinske pločice debljine od 0,4 do 3,6 mm. Razina difuzije bila je obrnuto proporcionalna debljini dentina koji se sastoji od dentinskih tubula. Toksičnost se smanjivala povećavanjem debljine dentina. Pri debljini većoj od 0,5 mm, toksičnost se smanjila za 75 posto, a iznad 1 mm toksičnost je pala za 90 posto (46). Stoga je debljina dentina kod IPP-a odlučujući čimbenik za kontrolu toksičnosti adhezivnih sustava.

Zaključci

I samojetkajući i dvokomponentni adhezivni sustavi imaju veliku citotoksičnost koja se smanjuje povećanjem razrjeđenja.

Adhese je imao najveću biokompatibilnost među testiranim adhezivima i najmanju citotoksičnost. Sljedeći je bio Excite s umjerenom citotoksičnošću. Xeno je imao veliku citotoksičnost, a za Prime & Bond citotoksičnost je bila najveća jer je njegova glavna komponenta UDMA.

Potrebna su daljnja istraživanja kako bi se ustanovilo koja je komponenta materijala odgovorna za štetne učinke na stanice. Trebalo bi uzeti u obzir i druga fizičko-kemijska svojstva koja bi mogla utjecati na uspješnost terapije.

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Sukob interesa

Nije naveden.

other authors (11), who have observed that Xeno shows less cytotoxicity as it has a lesser tendency to degrade, has more stable molecules and does not contain HEMA or bis-GMA.

Camphoroquinone (CQ) may be another cause of dentin adhesive cytotoxicity, being the most frequently used photoinitiator (44). This substance was present in all the materials that were evaluated (Table 1) and could affect cell metabolism, a possible mechanism provoking negative clinical and subclinical responses (42).

With regard to control samples, pH was seen to be a nonspecific variable that influenced the total cell viability. Thus, viability was seen to reduce due (partly) to this factor rather than the specific toxicity of the material; for this reason, the results obtained could achieve greater reliability by controlling this variable.

The thickness of the Dentin can have an effect on both the concentration and quantity of the adhesive reaching the pulp area. Hamid and Hume (45) investigated into the influence of dentin thickness on the level of penetration by the resin monomers in bonding agents after 24 hours incubation, testing dentin slices of 0.4-3.6 mm thickness. The diffusion rate was inversely proportional to the area of dentin consisting of dentinal tubules. Toxicity decreases as dentin thickness increases; if it is greater than 0.5 mm, toxicity is reduced by 75%, and if greater than 1 mm, toxicity falls by 90% (46) Therefore, dentin thickness in IPC is a determining factor for controlling the toxicity of adhesive systems.

Conclusions

Both self-etching and two-step adhesive systems show high cytotoxicity, which decreases as dilution increases.

Adhese presented the highest biocompatibility among the adhesives that were evaluated, and the lowest cytotoxicity. Next in order was Excite, found to present moderate cytotoxicity. Xeno presented high cytotoxicity, but Prime and Bond were found to display the highest cytotoxicity, as UDMA is its main component. Further studies are needed to determine which of the components of the material are responsible for harmful effects on cells. Such studies will need to take into account other physical and chemical properties of adhesives, which could affect the successful treatment.

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Conflict of Interest

None declared

Abstract
In many cases, the indirect pulp treatment (IPT) is an acceptable treatment for deciduous teeth with reversible pulp inflammation. Various medicaments have been used for IPT, ranging from calcium hydroxide and glass ionomers to dentin adhesives. **Objective:** This *in vitro* trial aimed to measure cytotoxicity in a cell culture, comparing the following four adhesives: Xeno® V (XE), Excite® F DSC (EX), Adhese® OneF (AD) and Prime & Bond NT (PB). **Materials and methods:** The adhesives were prepared according to the manufacturer's instructions. After 24 hours of exposure, the cell viability was evaluated using a photometrical test (MTT test). Data were subjected to analysis of variance (ANOVA). **Results:** Adhesives, the main component of which was 2-hydroxyethyl methacrylate (HEMA), were found to be less cytotoxic, while those that included the monomer urethane dimethacrylate (UDMA were the most cytotoxic) in their composition. The effects on cell viability assay varied between the adhesives assayed with statistically significant differences. **Conclusions:** The results may support the argument that Adhese® OneF is the least cytotoxic of the adhesives assayed, and may be considered as an adhesive agent for indirect pulp treatment. However, Prime and Bond NT showed a reduced biocompatibility under the same conditions.

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Dentin Bonding Agents; Cytotoxicity; Biocompatibility; Dental Pulp Capping

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